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# Determination of 2-Acetylaminofluorene Adducts by Immunoassay

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Antisera elicited in rabbits were used in radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) to determine femtomole quantities of deoxyguanosin-(8-yl)-acetylaminofluorene (dg-8-AAF) and deoxyguanosin-(8-yl)-aminofluorene (dg-8-AF). These adducts have been monitored in liver and kidney DNA of male Wistar-Furth rats fed 0.02% or 0.04% 2-acetylaminofluorene (2-AAF) either continuously or for a limited time followed by an interval on control diet. After 24 hr of 0.02% 2-AAF feeding, substantial levels of binding (80 fmole/μg DNA) were observed in liver DNA and increased with time, reaching a plateau of approximately 230 fmole/μg DNA at 30 days and thereafter. During the first week of continuous feeding about 80% of the total C-8 adducts in the liver DNA were deacetylated (dG-8-AF). By 25-60 days, dG-8-AF represented 97-100% of all C-8 adducts as measured by RIA and confirmed by HPLC. Values for C-8 adduct formation in kidney DNA were severalfold lower than in liver and dG-8-AF represented >90% of C-8 adducts at all times studied.

In removal or repair experiments, rats were fed 2-AAF for 3, 7 or 28 days, the 2-AAF diet was discontinued and the liver adducts assayed after intervals on control diet. When dietary 2-AAF administration was for 3 or 7 days, removal of adducts was efficient and almost complete by 28 days on control diet, with preferential retention of dG-8-AF. However, when dietary 2-AAF administration was for 28 days, adduct levels were higher, the repair capacity was saturated and the removal of C-8 adducts was not complete after control diet for a 28-day interval. In a preliminary experiment when [$^3$H]-2-AAF was fed for 3 days, after 25 days of 0.02% 2-AAF, the rates of newly formed adduct formation and removal were similar to those observed for the initial 3 days of 2-AAF feeding. These results demonstrate the predominance and persistence of dG-8-AF in liver and kidney DNA of 2-AAF-fed rats and suggest that the repair capacity of the whole rat liver was not diminished after 1 month of 2-AAF feeding.

## Introduction

The development and characterization of highly avid antibodies for carcinogen-DNA adducts has made possible the determination of femtomole ($10^{-15}M$) quantities of such adducts by sensitive immunoassays including radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (*1*). Antiguanosin-(8-yl)-acetylmainofluorene, elicited in rabbits, has been utilized to quantitate carcinogen-DNA adducts by immunological assays and the results compared favorably to other analytical procedures (*2, 3*). The antiserum is specific of the acetylated and deacetylated C-8 adducts of 2-acetylaminofluorene (2-AAF) with DNA and does not cross-react with the minor adduct, 3-deoxyguanosin-($N^2$-yl)-acetylaminofluorene (dG-$N^2$-AF), the ring-opened adduct 1[6-(2,5-diamino-4-oxypyrimidinyl-$N^6$-deoxyriboside)]-3-(2-fluorenyl)urea, (diamino Py-Fu) (M. Poirer, unpublished observations), the carcinogen alone or DNA (*4, 5*). The C-8 adducts, *N*-deoxyguanosin-(8-yl)-acetylaminofluorene (dG-8-AAF) and *N*-deoxyguanosin-(8-yl)-aminofluorene (dG-8-AF), comprise the major (approximately 90%) proportion of binding products formed upon interaction of 2- AAF, or its activated derivative *N*-acetoxy-2-acetylaminofluorene (N-Ac-AAF) with DNA *in vivo*, including cultured cells (*6-9*) and whole animals (*6, 10-14*). We have utilized RIA to quantitate formation and removal of guan-8-yl (C-8) adducts in liver and kidney DNA of male rats fed 2-acetylaminofluorene during a period of 2 months. It has been possible not only to deter-

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mine levels of bound product but also to distinguish between the acetylated and deacetylated C-8 adducts. The immunological approach is uniquely suited to investigation involving longterm chronic exposure, since it obviates the necessity for a radioactive carcinogen, can distinguish between different adducts of the same carcinogen and is sensitive enough to determine adducts in the range of one per $10^7$ nucleotides.

# Materials and Methods

## Animals and Diets

Young adult male Wistar-Furth or Fischer rats, weighing 130-150 g, were obtained from Microbiological Associates, Bethesda, MD, and three to four animals were housed in hanging wire or plastic cages. All animals were maintained on a 12-hr light cycle (light period: 7:00 A.M.-7:00 P.M.), permitted free access to tap water, and fed the standard control diet *ad libitum* until they reached a mean body weight of 185-188 g (inclusive) with a standard deviation of no more than ± 8 g.

All animals on a control diet received the purified, semisynthetic basal diet Bio-Mix No. 101 (Bio-Serv, Inc., Frenchtown, NJ). The composition of the diet has been described (*15*).

2-AAF (m.p. 192-196°C, Aldrich Chemical Co., Milwaukee, WI) was added to the control diet (Bio-Mix No. 101) at a concentration of 0.02% (w/w). [$^3$H]2-AAF, with a specific activity of 219 mCi/mmole, was kindly provided by Dr. F. A. Beland.

## Preparation of DNA from Rat Tissues

Tissues were homogenized and samples were prepared for cesium chloride isopycnic centrifugation as previously described (*16*). Each tube of the fractionated gradients was read at $A_{260}$, and the DNA peaks were dialyzed against deionized water.

## Radioimmunoassay

Procedures for the synthesis of all adducts for both immunization and RIAs, details of the immunization procedure and characterization of antibody specificity have been previously described (*2, 4, 5*). DNA samples for RIA were denatured and hydrolyzed by $S_1$ or $P_1$ nuclease. The $P_1$ nuclease (15 μg, Calbiochem, La Jolla, CA) was incubated with 250 μg DNA in 0.025 *M* sodium acetate (pH 5.6) and 0.001 *M* $ZnSO_4$ for 3 hr. Procedural details for the $S_1$ hydrolysis and performance of the RIAs have been given (*2, 8, 14*).

Two types of RIA have been employed in experiments described in this report. First, equal amounts of the same DNA were assayed simultaneously in two assays using [$^3$H]G-8-AAF and [$^3$H]G-8-AF as tracers, to approximate the proportion of each C-8 adduct. Secondly, the competition of increasing amounts of modified DNA against [$^3$H]G-8-AAF as tracer was assessed. When standard unlabeled dG-8-AF competes against [$^3$H]G-8-AAF at an antibody dilution of 1:2400, a saturation of the inhibition profile is observed at about 40% inhibition. Standard mixtures of acetylated and deacetylated adducts have been shown to saturate at higher percentage inhibition levels, the magnitude of increase being proportional to the percentage of dG-8-AAF in the mixture (*5*). The proportion of acetylated and deacetylated C-8 adduct in the unknown samples has been determined by assay with the appropriate standard curves after evaluation of the above DNA profiles.

## High Pressure Liquid Chromatography

HPLC was performed on hydrolyzates of DNA obtained from livers of rats fed [$^3$H]-2-AAF. This assay was performed by Ms. Nancy F. Fullerton in the laboratory of Dr. F. A. Beland according to previously published procedures for DNA hydrolysis and HPLC analysis (*6, 12*).

# Results

## Formation of Guan-8-yl Adducts in Liver and Kidney DNA of Male Wistar-Furth Rats Fed 0.02% and 0.04% 2-AAF

During the 60 days of chronic feeding, there was a rapid initial rise in adduct formation followed by a plateau of C-8 adduct levels in both liver and kidney DNAs. The plateau was reached by 1 month of 2-AAF feeding (Fig. 1). In animals fed 0.04% 2-AAF, the rate of liver DNA adduct formation was greater than the rate of adduct formation for the lower dose, but the magnitude of the plateaus appeared similar (Fig. 1*A*). At early times (24-72 hr) after the initiation of 2-AAF feeding, levels of DNA-bound C-8 adduct in the liver were 30-50% of the maximum plateau values, indicating that the formation of C-8 adduct occurred rapidly in liver DNA upon chronic feeding.

Levels of DNA-bound C-8 adduct in kidneys of the same animals paralleled those in the liver, but were 5- to 10-fold lower in magnitude, (Fig. 1*B*). The plateau occurred at a similar time, and adduct levels were higher with 0.04% 2-AAF than with the lower dose.

No adduct was detected in liver or kidney DNA from control animals at any time studied (Fig. 1).

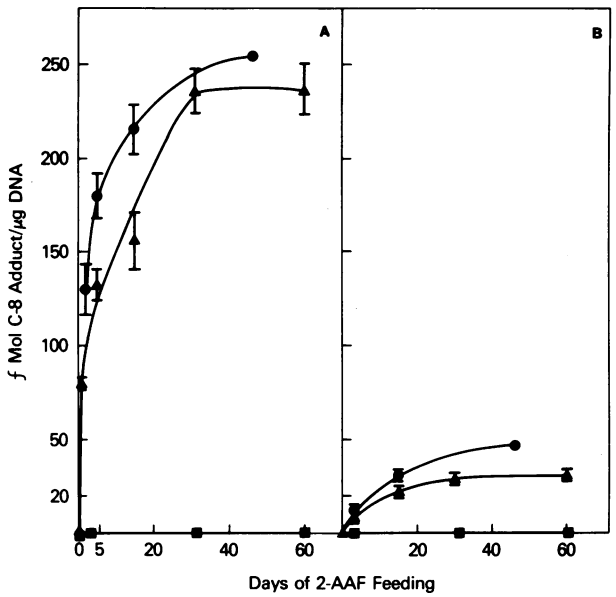


FIGURE 1. Formation of C-8 adducts in (*A*) liver DNA and (*B*) kidney DNA of male WF rats fed (▲) 0.02% 2-AAF, (● 0.04% 2-AAF or (■) control diet continuously for periods of time up to 60 days. DNAs were prepared by CsCl buoyant density centrifugation, heat-denatured and hydrolyzed with $S_1$ nuclease before assay by RIA. Each point represents mean ± S.E. of three animals.

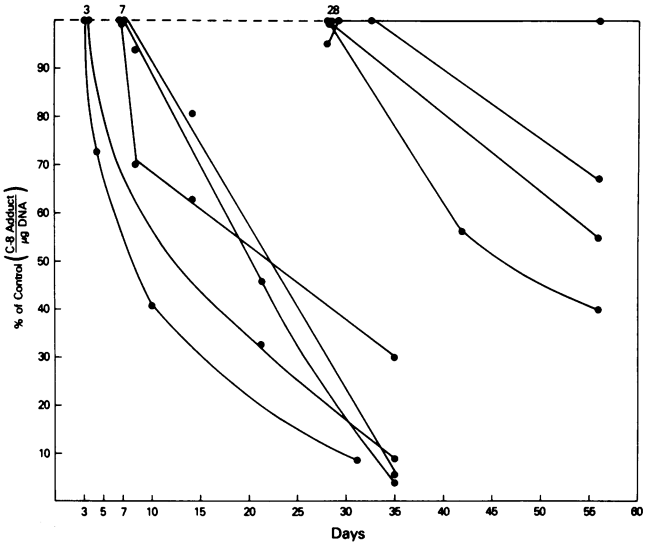


FIGURE 2. Removal of C-8 adducts (expressed on the ordinate as % of control) from liver DNA during 28 days on control diet (— —) subsequent to 3, 7 or 28 days of feeding 0.02% or 0.04% 2-AAF (--). DNAs were prepared and assayed as in Figure 1 except that some experiments employed $P_1$ nuclease hydrolysis. Each point represents the mean from two to four rats, and all rats were male WF with the exception of one experiment in which Fischer F344 rats were fed 2-AAF for 3 days.

## Removal of C-8 Adducts from the Livers of 2-AAF-Fed Rats after Intervals on Control Diet

When 2-AAF was administered orally for short periods of time, (3 or 7 days) and the animals given control diet for 1 month subsequently, C-8 adduct removal appeared to be efficient and complete. In four out of five experiments adduct levels were decreased by ≥90% after 28 days on control diet (Fig. 2). A very different phenomenon was observed when animals were fed 0.02% 2-AAF for 28 days (Fig. 2). In one out of four experiments there was no decrease in the level of C-8 adducts by 28 days on control diet. In the remaining three experiments, removal was between 35 and 60% at this time. Thus we were presented with an enigma. On the one hand, the amount of total C-8 adduct reached a plateau, indicating a possible steady state between formation and removal, and on the other hand there was apparently less removal after 28 days of feeding 0.02% 2-AAF.

To further investigate this phenomenon, an experiment was designed in which four animals were fed 0.02% 2-AAF for 25 days, followed by feeding [$^3$H]-2-AAF (0.02%) for 3 days. At 28 days, two animals were sacrificed, and the remaining two were given control diet for another 28 days. Adduct levels after the initial 28-day period are represented in Figure 3 by the solid squares and triangles (■, ▲), the former being adducts determined by radioactivity and the latter being values for total C-8 adduct measured by RIA. In a parallel experiment rats were fed unlabeled 2-AAF for 3, 14 or 28 days, and some animals were sacrificed after an additional 14 and 28 days on control diet. Although this study is preliminary, Figure 3 suggests that the rates of adduct formation and removal were almost the same at 3 and 25 days of 2-AAF feeding. The similarity in the rates of adduct formation can be clearly seen by comparing the (●) and (■) curves in Figure 3. The similarity in the rates of adduct removal is somewhat more subtle, but when calculated as adducts removed per day, by 14 days on control diet animals fed 0.02% 2-AAF for 3 days had removed 6.4 femtomoles/day and animals fed for 28 days had removed 6.1 femtomoles/day (Fig. 3). For the same animals at 28 days on control diet, 4.3 fmole was removed per day, and in the radioactive animals 3-3.5 fmole was removed per day as measured by RIA and radioactivity. These calculations provide only a rough approximation, since there were too few points for well-defined curves but all these rates of removal are probably not significantly different. Therefore the amount of adduct remaining after 28 days on control diet would appear to be a reflection of the amount of C-8 adduct pre-

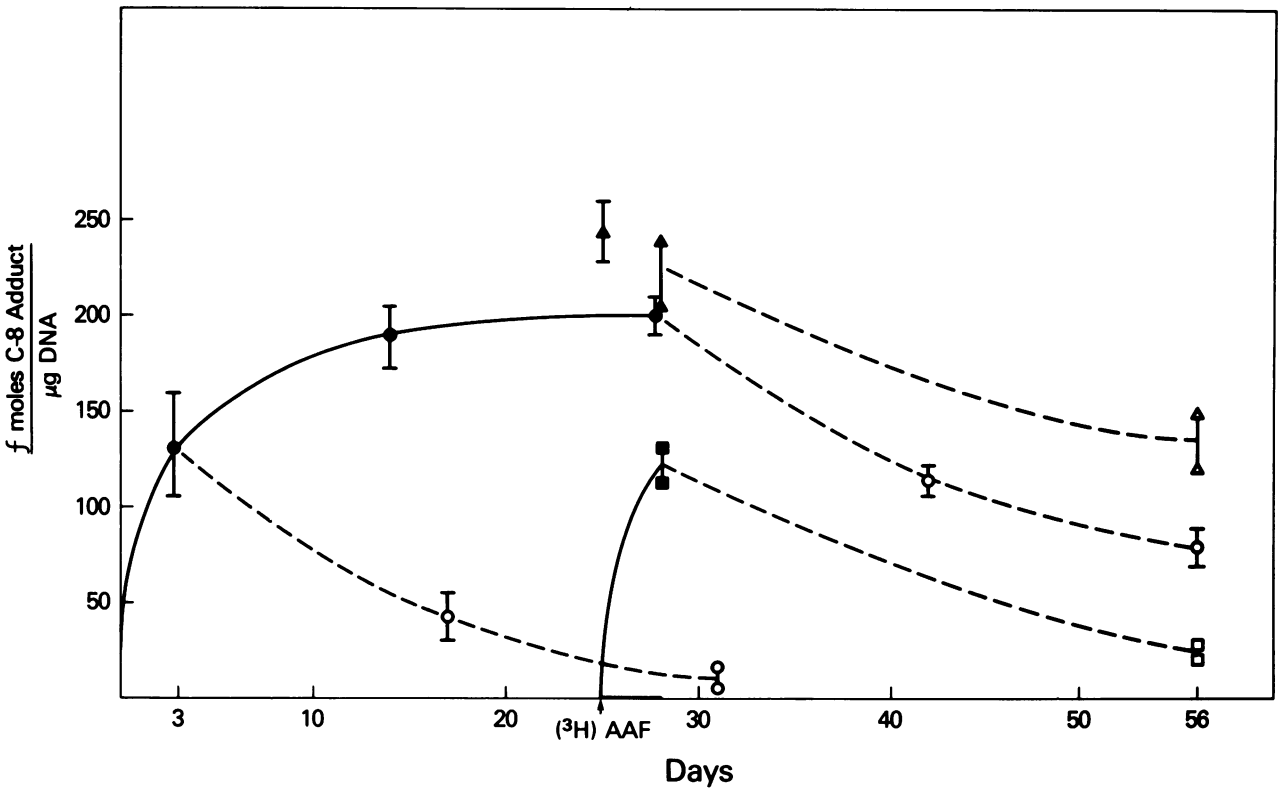


FIGURE 3. Formation (—— , ●, ▲, ■) and removal (--, ○, △, □) of C-8 adducts in male WF rats fed 0.02% 2-AAF. Seven animals were fed 0.02% 2-AAF for 25 days, at which time three were sacrificed (▲, 25 days), four were fed 0.02% [$^3$H]-2-AAF (219 mCi/mmole) for 3 days, at which time two were sacrificed (▲, 28 days) and two were given control diet for a subsequent 28 days (△). Values for total C-8 adduct obtained by RIA from these liver DNAs are represented by ▲ and △; values for the newly formed (25-28 days) radioactive adducts in the same animals are represented by ■ and □. In a parallel experiment, values for adduct formation (●) and removal (○) are shown for liver DNA from male WF rats fed 0.02% 2-AAF for 3, 14 and 28 days. DNAs were prepared as indicated for Figure 1, except that $P_1$ nuclease was used for DNA hydrolysis, and values, represented are either mean ± S.D. for three animals or individual points for two animals.

sent upon cessation of 2-AAF feeding. For example, of the radioactive 2-AAF, only 20% remained after 28 days on control diet when the original adduct level was 120 fmole/μg DNA. Of the total C-8 adduct in the same animals (measured by RIA) 61% remained, from an original adduct level of 220 fmole/μg DNA. Yet both groups of animals had removed about 100 fmole/μg DNA during this time.

Kidney DNAs were assayed from the same animals shown in Figure 3, and the results (not shown) exactly paralleled the data from liver except that the maximum plateau values for C-8 adducts in kidney were 62 fmole/μg DNA.

## Proportions of Acetylated and Deacetylated C-8 Adducts in Liver DNA During Feeding of 2-AAF With or Without a Subsequent Interval on Control Diet

Significant differences were observed in the proportions of the acetylated and deacetylated C-8 adducts at various times after the initiation of 2-AAF feeding. For both 0.02% and 0.04% 2-AAF at days 1, 3, 5 and 7, the DNA profiles with [$^3$H]G-8-AAF, and comparison with standard curves indicated that about 20% of the C-8 adducts were acetylated and 80% were deacetylated (Table 1 and Fig. 4). With continuous feeding there was clearly a progression toward a higher proportion of deacetylated C-8 adduct, since liver DNA from animals fed for 25 and 60 days all showed saturation profiles similar to dG-8-AF and hydrolyzed AF-DNA assayed simultaneously (Fig. 4). DNAs which saturate at the same percentage inhibition as the deacetylated standards have been shown to be totally deacetylated, and DNAs which saturate slightly (5-7% inhibition) higher than dG-8-AF in the same assay have been shown by linear regression analysis to contain 3% dG-8-AAF and 97% dG-8-AF (*19, 20*). In liver DNAs from rats fed 2-AAF for 25-60 days 0-3% of the C-8 adduct was the acetylated form (Table 1 and Fig. 4)

Table 1. Proportions of acetylated and deacetylated adducts in rat liver DNA.

| Days of feeding | | Adducts, % | |
|---|---|---|---|
| AAF | Control diet | dG-8-AF | dG-8-AAF |
| 0.02% AAF | | | |
| 3 | 0 | 80 | 20 |
| 15 | 0 | 80 | 20 |
| 25-31 | 0 | 97-100 | 0-3 |
| 60 | 0 | 97 | 3 |
| 0.04% AAF | | | |
| 3 | 0 | 80 | 20 |
| 15 | 0 | 97 | 3 |
| 46 | 0 | 97 | 3 |
| 0.02 or 0.04% AAF | | | |
| 7 | 7 | 100 | — |
| 7 | 28 | 100 | — |
| 28 | 7 | 97-100 | 0-3 |
| 28 | 28 | 97-100 | 0-3 |

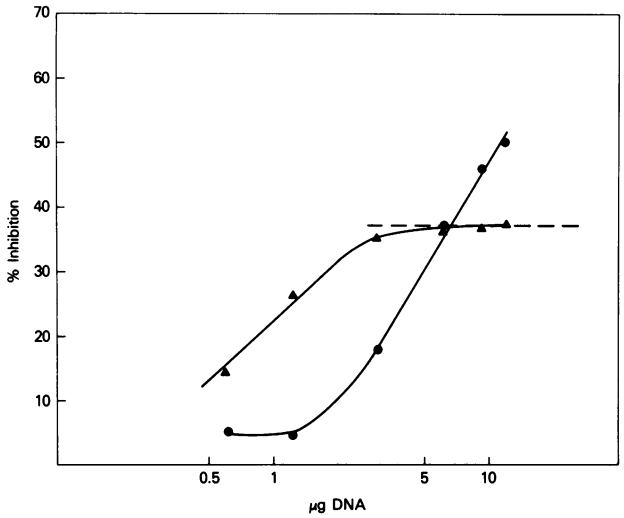


FIGURE 4. DNA profiles in RIA of increasing concentrations of liver DNA samples from male WF rats fed 0.02% 2-AAF for (●) 3 days or (▲) 7 days (--) percent inhibition for standard dG-8-AF saturation in the same assay is indicated. Samples were prepared as indicated in Figure 1 except $P_1$ nuclease digestion was employed without denaturation for DNA hydrolysis.

and 97-100% was dG-8-AF. Similar criteria indicated that the liver DNAs from rats fed 2-AAF followed by 1-4 weeks of feeding control diet contained 97-100% of C-8 adducts as deacetylated (Table 1).

The experiment (Fig. 3) in which rats were fed [$^3$H]2-AAF for 3 days after 25 days of unlabeled 2-AAF, provided a unique opportunity to compare analysis of adduct formation by RIA and by HPLC. RIA profiles indicated that DNAs from animals fed 2-AAF for 28 days contained dG-8-AF as 97-100% of the total C-8 adduct (Table 1). The HPLC confirmed the presence of one major C-8 adduct peak dG-8-AF, (Fig. 5), and only 1.8-2.9% of the total C-8 radioactivity cochromatographed with the dG-8-AAF standard. In addition there was no evidence for significant quantities of dG-N$^2$-AAF or diamino-Py-FU in this profile. After 25 days on control diet there was only the dG-8-AF peak which was, this time, much reduced in size (Fig. 5).

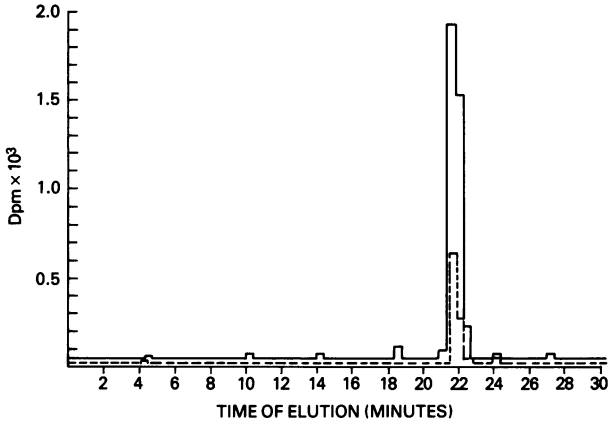


FIGURE 5. High pressure liquid chromatography (HPLC) profiles of radioactivity in liver DNA from male WF rats (— —) fed 0.02% 2-AAF for 25 days followed by 0.02% [$^3$H]-2-AAF (219 mCi/mmole) for 3 days) (--) Parallel animals (--) were additionally fed control diet for 28 days subsequently. DNAs were hydrolyzed with DNAse, alkaline phosphatase and venom phosphodiesterase, and hydrolysis was 90% complete. The largest peak in both profiles eluted in the same position as standard dG-8-AF adduct (22 min). In the experimental profile (——), the small peak at 18.3 min corresponded to the elution of standard dG-8-AAF or G-8-AF. Since this DNA was prepared by CsCl gradient centrifugation this is probably primarily the deoxy adduct. The base line for the control profile (--) was plotted lower by 25 DPM for better resolution of the two lines.

## Discussion

Determination of guan-8-yl adducts of 2-acetylaminofluorene in liver and kidney DNAs of rats fed the parent carcinogen has been accomplished utilizing sensitive RIAs with a specific G-8-AAF antiserum, and analytical HPLC after hydrolysis of radioactive DNAs. The combination of both procedures has yielded new information concerning the formation and removal of 2-AAF-DNA adducts in rat liver during 2 months of chronic exposure to a carcinogenic feeding regimen.

Binding levels for total C-8 adducts in the continuous feeding experiments increased rapidly during the first few days of feeding, reaching a plateau above 200 fmole/μg at about 1 month. A similar phenomenon has been observed by Irving (*17*), who fed 0.04% radiolabeled 2-AAF to male rats for 8 weeks and monitored total DNA adducts (including the N$^2$ adduct which we are unable to measure). Both Irv-

ing (*17*) and Szafartz (*18*) found plateau levels for total adducts in the range of 100-400 fmole/μg DNA upon feeding radioactive 2-AAF or N-OH-AAF at doses between 0.016% and 0.04%. Given the potential sources of variability in carcinogen feeding regimens, it is likely that there is biological significance to these similar plateau values for 2-AAF bound to liver DNA during chronic exposure. In future studies with an initiation-promotion regimen, one could perhaps establish binding levels necessary (but not sufficient) for tumorigenesis.

With short-term feeding or intraperitoneal injection of radiolabeled 2-AAF or N-OH-AAF, several investigators have observed efficient removal of adducts from liver DNA (*10, 12, 18-20*), similar to the data shown in Figure 2 at 3 and 7 days. However, inability of the liver to complete removal of dG-8-AF in DNA after long-term 2-AAF feeding (Fig. 2) has not been previously reported. The experiment with [$^3$H]-2-AAF (Fig. 3) was designed to determine the rate of the newly formed adduct at later times during chronic feeding and also to explore the rate of removal. Even though these results are preliminary and a more extensive time course is needed, these data indicate that the rates of formation and removal of C-8 adducts after 28 days of 0.02% 2-AAF in the diet are similar to those after 3 days of 2-AAF feeding. Thus, after 28 days of 2-AAF feeding there does not appear to be an alteration in removal rates. The adduct levels at 28 days were about 2-fold higher than after 3 days of 2-AAF feeding and the time required for 90% removal at a fixed rate would appear to have been doubled. This experiment, if reproducible, raises many more questions than previously anticipated. It is difficult to reconcile the fact that at both 3 and 28 days the rate of adduct formation was 3- to 4-fold higher than the rate of removal while the adduct levels reach a plateau at approximately 1 month of feeding. Also, how is it possible to incorporate the information that the newly formed adduct constituted half of the C-8 adduct in total liver DNA at 28 days of feeding and appeared to be preferentially removed, since 80% was gone during the removal period? These findings might be explained if binding of carcinogen occurred unevenly in the liver and/or caused cell selection to occur. Such a situation would require that the earliest binding regions would undergo a loss of metabolic capability, thus limiting further adduct formation while still maintaining a fixed removal capacity. In other areas adduct accumulation would occur later during the course of exposure and would have the normal removal capacity. Experiments designed to test this hypothesis will include analysis by immunofluorescence techniques as well as RIA and HPLC.

By RIA we have shown that the deacetylated adduct, dG-8-AF, increased proportionally both with time on the 2-AAF diet, and subsequent feeding of control diet. At early times (1, 3, 5, 7 days) about 80% of the C-8 adduct was deacetylated, and at later times (25-60 days) 97-100% was dG-8-AF. In the repair experiments where animals were fed 2-AAF for 3 or 7 days, there was a marked progression from about 80% dG-8-AF initially to 100% dG-8-AF after a week on control diet. These observations were confirmed by HPLC which showed the presence of one major adduct, dG-8-AF, after 28 days on 0.02% 2-AAF and also after a subsequent 28 days on the control diet. Since Irving has demonstrated a decrease in rat liver sulfotransferase with 2 weeks of 2-AAF feeding (*21*), it was not unexpected to find that neither dG-8-AAF or dG-N$^2$-AAF were formed in rat liver DNA at this time. Thus it would appear that accumulation of dG-8-AF may be the most persistent sequela of chronic 2-AAF feeding where effects on DNA are concerned. The kinetics of dG-8-AF removal have not been investigated in female rats; the adduct formation follows a pattern similar to that observed in the males (*17*) although dG-8-AF is the only adduct formed (*12*). Since female rats are more resistant to liver tumor formation, presence of the adduct alone probably does not constitute an effect sufficient for tumorigenesis. On the other hand, the acetylated 2-AAF adducts (dG-N$^2$-AAF and dG-8-AAF) which are formed only in male rats and only at early times during chronic 2-AAF feeding may contribute to the initiating effects of 2-AAF, while accumulation of dG-8-AF may participate somehow in promotion.

In further experiments a combination of immunological and analytical procedures should yield more complete information concerning the accumulation of DNA adducts and the biological responses of the liver during hepatocarcinogenesis by 2-AAF.

We are particularly grateful for the helpful discussions and suggestions of Dr. S. Yuspa. Drs. I. B. Weinstein and S. Blobstein supplied us with the original antigen for these studies. The [$^3$H]-2-AAF was the gift of Dr. F. A. Beland. Ms. Nancy F. Fullerton of the same laboratory hydrolyzed the radioactive DNAs and performed the HPLC. Dr. E. Kriek provided diamino-Py-FU which was used in competition studies with anti-G-8-AAF. The excellent technical assistance of Marc Dubin, Curtis Thill and Elroy Patterson is also appreciated, as is the secretarial expertise of Maxine Bellman.

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